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INTRACELLULAR pH IN EARLY *XENOPUS* EMBRYOS: ITS EFFECT ON CURRENT FLOW BETWEEN BLASTOMERES

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SUMMARY

1. Electrophysiological techniques were used to monitor the flow of electric current from one cell to the next in *Xenopus laevis* embryos between the 4-cell and early blastula stages of development. Intracellular pH and blastocoel pH were determined using pH-sensitive micro-electrodes.

2. The resting intracellular pH was $7{\cdot}74 \pm 0{\cdot}02$ (S.E. of mean, $n = 29$); there were no systematic differences between developmental stages. Blastocoel cavity pH was $8{\cdot}4 \pm 0{\cdot}06$ (S.E. of mean, $n = 10$). The intracellular buffer value was 18 m-equiv. H^+/pH unit per litre.

3. In embryos treated with bicarbonate buffered Holtfreter solution equilibrated with 100% CO_2 the intracellular pH fell to $6{\cdot}3 \pm 0{\cdot}17$ (S.D., $n = 8$). The membrane potential fell and the input resistance increased. The size of the effect on membrane potential and input resistance varied.

4. From the 32-cell stage onwards current flow from one cell to the next was abolished when the intracellular pH fell to below 6·5; the effect was rapid in onset and completely reversible. At cleavage stages of development lowering intracellular pH with CO_2 had no effect on current flow from cell to cell.

5. The relationship between intracellular pH and current flow from cell to cell was sigmoid and covered between 0·2 and 0·4 pH units. The pH at which current flow was completely abolished ranged from 6·85 to 6·4.

6. Alterations in extraembryonic pH over the range 5·8–7·5 had no effect on any parameter measured.

7. We conclude that lowering the intracellular pH increases the resistance of both non-junctional and junctional membranes. The data do not allow us to extract the pH-junctional conductance relationship.

8. Variations in intracellular pH may provide a useful tool for the study of the functional role of direct cell to cell communication in both adult organs and early embryos.

* Intracellular pH measurements were made in the laboratory of R. C. Thomas.

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INTRODUCTION

The cells of many tissues are able to exchange ions and small metabolites through a direct cell to cell pathway. Thus, current injected into one cell affects the membrane potential of its neighbours. When injected into one cell, fluorescent dyes such as Lucifer yellow, Procion yellow and fluorescein are seen to move into adjacent cells, and cells in tissue culture exchange small metabolites such as thymidine monophosphate. Evidence for direct cell to cell communication has been obtained in, for example, epithelial cells, bladder cells and *Drosophila* larvae salivary gland (Loewenstein, Socolar, Higashino, Kanno & Davidson, 1965), liver cells (Penn, 1966), pancreatic acini (Petersen & Ueda, 1976), mammalian salivary gland cells (Hammer & Sheridan, 1978). Similarly, cells within embryos of many species have been shown to be in ionic communication with each other (Potter, Furshpan & Lennox, 1966; Ito & Loewenstein, 1969; Palmer & Slack, 1970; Tupper & Saunders, 1970). It is likely that the transfer of molecules and small ions from one cell to the next takes place at the intercellular junction identified in the electron microscope as the 'gap' junction (Gilula, Reeves & Steinbach, 1972).

Considerable information about the permeability of this pathway has now accumulated, particularly in differentiated cells (see Bennett, 1978) but the mechanism by which the permeability of the gap junction is controlled remains obscure. Similarly, the functional role of such direct cell to cell communication has not been determined in adult inexcitable tissues or in early embryos. It would clearly be helpful for elucidation of the functional role of such direct intercellular pathways if it were possible to abolish simply and reversibly communication between cells and observe the consequences. Rose and Loewenstein (1976) have shown that when the intracellular concentration of ionized calcium rises within cells of the *Chironomus* salivary gland, either because of damage or when calcium is injected intracellularly, the permeability of the direct pathway falls. Injection of calcium ions allows the isolation of a single cell from its neighbours, but, particularly in early embryos, only complete abolition of the pathway between all communicating cells is likely to provide the necessary tool for study of the function of direct cell to cell communication.

The permeability of cell surface membranes has long been known to be sensitive to alterations in pH and it seemed possible that the membranes involved in creating the cell to cell communication channel might similarly be sensitive to alterations in environmental pH. Since the gap junction is only accessible from the intracellular surface of cells, alterations in intracellular pH were thought more likely to be effective.

This paper reports experiments to determine the effect of altering intracellular pH on ionic communication between cells of the early embryo of *Xenopus laevis*. Some measurements of intercellular pH, resting intracellular pH and buffer value of early embryonic cells are also included. A preliminary report of some of these results has already appeared (Turin & Warner, 1977).

METHODS

Mature pairs of *Xenopus laevis* were induced to lay by injection of chorionic gonadotrophin (Organon, Ltd.). The embryos were staged according to the Life Table of *Xenopus* (Nieuwkoop & Faber, 1956); embryos between the 4-cell and early blastula stage were used. The jelly was removed mechanically with fine forceps and the embryo placed in a small depression in a wax filled bath through which solutions of the desired composition flowed. The composition of the solutions is given in Table 1.

TABLE 1. (All quantities in mM)

NaCl	KCl	$CaCl_2$	Buffer	CO_2 (%)	pH
60	1·2	4	10 (ACES* or ADA*)	—	6·5
60	1·2	4	10 (HEPES*)	—	7·5
20	1·2	4	40 ($NaHCO_3$)	5	7·5
55	1·2	4	10 ($NaHCO_3$)	20	6·4
50	1·2	4	20 ($NaHCO_3$)	40	6·4
40	1·2	4	30 ($NaHCO_3$)	60	6·4
30	1·2	4	40 ($NaHCO_3$)	80	6·4
20	1·2	4	40 ($NaHCO_3$)	100	6·3

* ACES = *N*-(2 acetamido)-2-amino ethane sulphonic acid, ADA = *N*-(2 acetamido)-2-iminodiacetic acid, HEPES = *N*-2-hydroxyethylpiperazine-N′-2 ethane sulphonic acid.

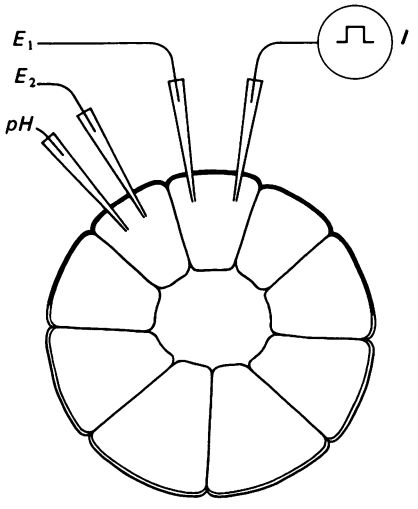

Fig. 1. Diagram to show the arrangement of the electrodes. E_1 and E_2 record the membrane potential and the electrotonic potentials V_1, V_2 produced by injection of intracellular current through the current passing electrode *I*. The pH-sensitive electrode is inserted into the same cell as E_2 which serves as a reference electrode for pH_i measurements.

Membrane potentials were recorded using intracellular micro-electrodes filled with 0·8 M-K citrate or 3 M-KCl (Tip potentials < -8 mV; resistance 20 MΩ). Rectangular hyperpolarizing current pulses, 10 or 1 sec long, were injected through a third micro-electrode and a fourth, pH-sensitive micro-electrode (Thomas, 1974) was also inserted. The arrangement of the electrodes is shown diagrammatically in Fig. 1. E_1 measured the membrane potential of one cell and the electrotonic potential produced by injection of current into that cell (V_1). E_2 recorded the mem-

brane potential and electrotonic potential (V_2) in an adjacent cell. The pH-sensitive electrode, which gave between 54 and 57 mV/pH unit, measured both pH_i and the membrane potential. It was inserted into the same cell as E_2, which served as a reference electrode for pH_i measurements. The signal from the pH electrode was fed into an Analog Devices 311J Varactor Bridge amplifier and the difference between this signal and the membrane potential (pH_i) obtained electronically. The response time of the pH electrode to alterations in H^+ concentration was about 15 sec and was diffusion limited (Thomas, 1974); the response time for voltage changes was determined by the RC properties of the electrode. To reduce the voltage time constant and eliminate capacitative artefact the pH electrode was screened by a driven shield; despite this precaution a lag remained between the voltage recorded by the pH electrode and E_2, which sometimes appeared on the pH_i trace as a small voltage artifact at the beginning and end of each current pulse (e.g. Fig. 4). Membrane potentials were recorded using conventional electrophysiological techniques. All parameters were displayed on an oscilloscope, and recorded on a pen recorder and on magnetic tape. The indifferent Ag:AgCl electrode made contact with the bath via an Agar–Holtfreter half cell. All experiments were done at room temperature (18–22 °C).

RESULTS

The average intracellular pH recorded from cells of early *Xenopus* embryos between the 4-cell and early blastula stage was $7{\cdot}74 \pm 0{\cdot}02$ S.E. of mean, $n = 29$). The values obtained varied from clutch to clutch, ranging from 7·62 to 7·9. There was less variation between embryos from the same clutch. No systematic difference was observed between developmental stages. This value for resting intracellular pH is high compared with that obtained in snail neurones (7·4: Thomas, 1974), heart muscle (7·02: Ellis & Thomas, 1976), skeletal muscle (7·07: Aickin & Thomas, 1977), barnacle muscle (7·35: Boron & Roos, 1976). The intercellular pH, measured at stages $6\frac{1}{2} - 7$ (64 – > 128 cell), when the blastocoel volume first becomes appreciable, was also high with a mean of $8{\cdot}4 \pm 0{\cdot}06$ (S.E. of mean, $n = 10$). As the embryo enters these stages the membrane potential rises to between -40 and -50 mV. Since the blastocoel potential is close to zero (Slack & Warner, 1973) this means that the distribution of hydrogen ions between the intracellular and intercellular compartments (the blastocoel) is close to that to be expected from the membrane potential. The intercellular and intracellular pH were not affected by alterations in pH between 5·8 and 7·5 of the solution bathing the embryo.

Fig. 2 shows the effect on the intracellular pH of superfusing the embryo with bicarbonate buffered solutions equilibrated with different percentages of carbon dioxide (for composition see Table 1). Treatment with 5% CO_2 brought the intracellular pH within the range recorded in other cells. Higher percentages of CO_2 produced a greater fall in intracellular pH, with the maximum response at 100% CO_2.

When the embryos were exposed to 5% CO_2 it was found that the initial fall in pH_i was followed by a gradual return to the baseline pH even though 5% CO_2/HCO_3^- buffered solution continued to flow through the bath. This suggested that H^+ ions were being pumped out from the cells during CO_2 exposure, as in other cells (Thomas, 1977). Return to ADA or ACES buffered solution always caused a transient increase in pH_i to above the baseline value. Thus, although it seems likely that in untreated embryos a H^+ ion pump is not working, since the ratio of H^+ ions across the membrane is relatively close to that to be expected from a passive distribution, a H^+ ion pump can be activated when $(H^+)_i$ is raised by CO_2 application.

The information given in Fig. 2 can be used to determine the intracellular buffering power of early amphibian embryonic cells. The intracellular pH during application

of a given partial pressure of CO_2 is governed by a form of the Henderson–Hasselbalch equation, making the assumption that within the time course of the measurements pH_i is dominated by entry of undissociated carbon dioxide:

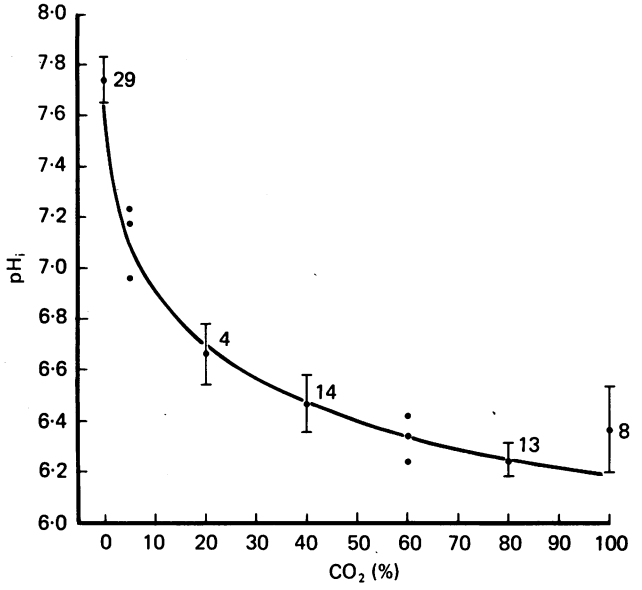

Fig. 2. The relation between intracellular pH and the % CO_2 equilibrated with HCO_3^- buffered Holtfreter solutions. Ordinate: pH_i abscissa: % CO_2. Where error bars are shown (± S.D.) the points give the mean. The figure next to the points give the number of measurements. When three measurements only were taken, all points are given. Continuous line drawn according to eqn. (1) for a buffer value of 18 m-equiv H^+/pH unit per litre (see text).

$$pH_1 \text{ final} = pK_a + \log[(\text{buffering power}).(pH_1 \text{ initial} - pH_1 \text{ final})/(0{\cdot}03 \times 7{\cdot}6 \times \%CO_2)]$$

where pK_a = pK of carbonic acid (6·1) and 0·03 the solubility constant of CO_2 at 20 °C. For calculation of the buffering power the initial $[HCO_3^-]_i$ was taken to be negligible and pH_i set at 7·7. The average pH change produced by 40 % CO_2 was used to calculate the buffer value; which came to 18 m-equiv H^+/pH unit per litre by numerical solution of the equation above. This calculated buffer value was then used to determine the continuous line drawn through the points of Fig. 2. This gives a good fit to the experimental results. The fit for 100 % CO_2 is not as good as lower CO_2 tensions, although the calculated line falls within one standard deviation of the mean. A number of these values were obtained on a batch of embryos which was not tested with lower percentages of CO_2. Since there was always considerable variability from batch to batch this could have biased the results.

When an intracellular micro-electrode is inserted into a cell of a *Xenopus laevis* embryo, a dense pigment ring always forms around the electrode at the site of insertion. This is probably due to Ca^{2+} dependent contraction of the contractile cortex which lies beneath the plasma membrane (Selman & Perry, 1970; Gingell, 1970; Baker & Warner, 1972). Whenever the embryo was treated with CO_2 it was noticeable that the pigment ring became less dense, and any cleavage in progress regressed temporarily.

The effect of CO_2 on electrical coupling

Fig. 3 shows the effect of superfusing an embryo at the 32-cell stage with a 100% CO_2/HCO_3^- buffered solution on current flow from cell to cell.

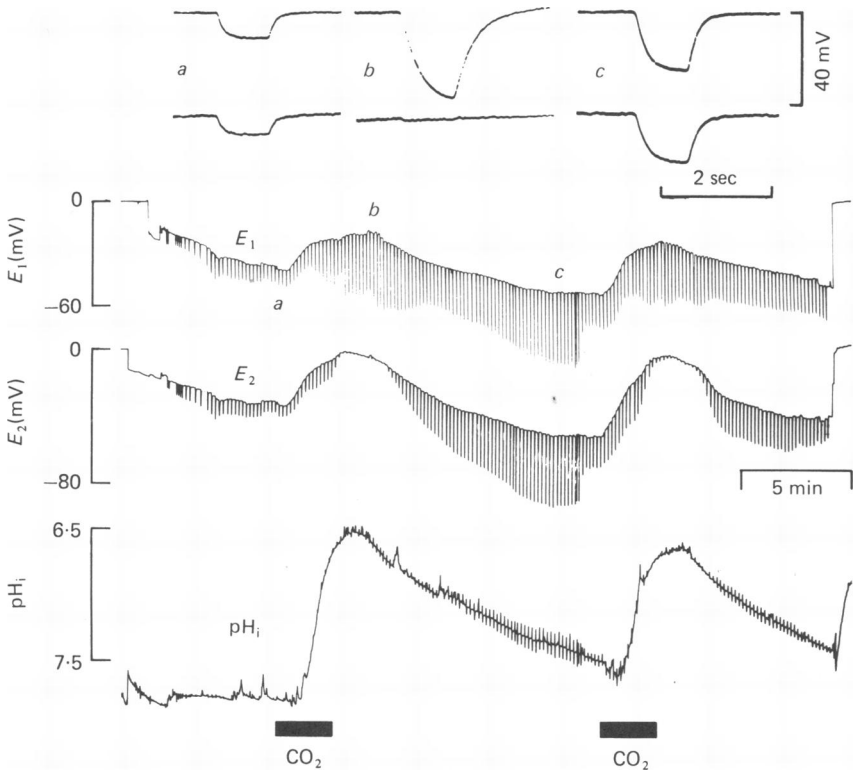

Fig. 3. The effect of 100% CO_2 on membrane potential, electrical coupling and intracellular pH. Embryo 32-cell stage. Upper panel: oscilloscope records of electrotonic potentials at points *a*, *b*, *c* indicated on slow pen records shown in lower panel. Lower panel: ordinates, membrane potential on E_1 and E_2, and pH_i. Hyperpolarizing current pulses 1 sec long. 40 nA reduced to 20 nA before second exposure to CO_2.

The pH-sensitive electrode was first inserted, followed by E_2, then E_1. Finally the current-passing electrode was inserted into the same cell as E_1 and electrotonic potentials appeared on the E_1 and E_2 trace. With all four electrodes inserted and the record stable, pH_i was 7·8, the potential at $E_2 - 35$ mV and at $E_1 - 42$ mV. The coupling ratio, given by the height of the electrotonic potential on E_2 divided by the height of the electrotonic potential on E_1 (V_2/V_1), was 0·64. Once the input resistance had reached a steady value the external solution was changed from an ACES buffered solution to one buffered with 100% CO_2/HCO_3^-. The increase in CO_2 level had three consequences. The membrane potential of both cells fell, reaching -17 mV at E_1 and 0 mV at E_2. The intracellular pH fell from its initial high value to 6·53. The input resistance recorded by E_1 rose 4·3 times and the electrotonic potential recorded by E_2 disappeared completely, showing that current was no longer flowing from cell to cell. Complete abolition of electrical coupling occurred 2·5 min after

admission of the CO_2 containing solution. As soon as uncoupling was complete the bathing solution was returned to ACES buffered Holtfreter solution. Over the next 12 min the intracellular pH, membrane potentials and electrotonic potentials recovered. The half time for the recovery of pH_i was 5 min. Both the membrane potentials and coupling ratio returned to values greater than those recorded before

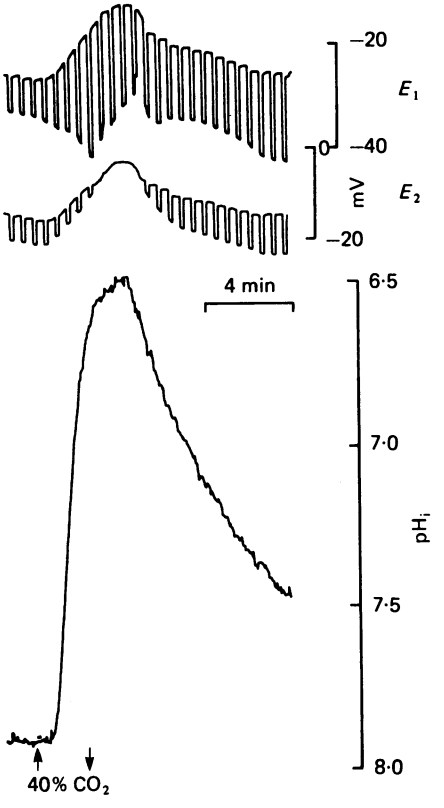

Fig. 4. The effect of 40% CO_2 on membrane potential, electrical coupling and intracellular pH. Embryo 64-cell stage. Hyperpolarizing current pulses 10 sec long. 5 nA pulse interval 30 sec.

exposure to CO_2. A second exposure to 100% CO_2 again produced uncoupling of adjacent cells, together with a fall in membrane potential and pH_i. The pH_i attained during the second exposure to CO_2, 6·62, was higher than that seen previously, perhaps reflecting activation of the H^+ pump during the first exposure to CO_2 (see Thomas, 1977). On a number of occasions the extracellular pH was changed from pH 7·5 to 5·8; there was no effect on electrical coupling. The accuracy of the record of the fall in pH was almost certainly limited by the response time of the pH-sensitive micro-electrode, but the recovery was much slower and the record suggests that the cells remain uncoupled as long as the intracellular pH remains below 6·8.

Inspection of Fig. 2 suggests that complete uncoupling of adjacent cells should also be achieved by much lower CO_2 tensions. Fig. 4 shows that solution buffered with 40% CO_2/HCO_3^- is equally effective. In this experiment the resting intracellular pH

was 7·9 and it fell to 6·5 while CO_2 containing solution flowed through the bath; this was accompanied by depolarization and complete abolition of current flow from cell to cell.

When the intracellular pH fell to below pH 6·5 current flow from one cell to the next was completely abolished. The intercellular fluid compartment of the embryo is isolated from the bathing solution by tight junctions situated at the external borders of the outermost cells. These junctions must be relatively impermeable to small ions, in order to maintain the observed NaCl concentration gradient between the bathing solution and the intercellular fluid (Slack, Warner & Warren, 1973). In such circumstances one would expect some current to flow from cell to cell through a restricted extracellular space as well as through a pathway mediated by gap junctions. If an increase in non-junctional membrane resistance occurs at the same time as the effect of CO_2 on the direct pathway then current flow through the blastocoel would be reduced. Nevertheless the fact that current flow from cell to cell is completely abolished by CO_2 treatment suggests that there must be some weakening of the intercellular seal at the tight junction. Similar considerations must also apply to Iwatsuki & Petersen's (1979) experiments on pancreatic acini, for here too cells are joined together by tight junctions at their apical surfaces, yet CO_2 completely abolishes intercellular current flow. The explanation for their finding that in the lacrimal gland current flow from cell to cell is reduced, but not completely abolished, may lie in a more effective tight junctional seal so that some current flow through the extracellular space remains. The *Chironomus* salivary gland also forms a three dimensional structure with the intercellular spaces isolated from the medium bathing the gland; it inevitably follows that when an increase in intracellular ionized Ca causes complete abolition of current flow from cell to cell (as in Rose & Lowenstein, 1975) that the intercellular seal must also have been broken.

In contrast to its effect on intracellular pH, the ability of CO_2 to prevent current flow from one cell the the next was critically dependent on the development stage of the embryo. Fig. 5. shows an experiment in which the effect of carbon dioxide on electrical coupling was tested on an embryo at the 4-cell stage. As at early blastula stages the initial pH was high, 7·8, and admission of 100% CO_2/HCO_3^- buffered solution produced a dramatic fall in intracellular pH to 6·4. Both E_1 and E_2 showed a relatively small depolarization, probably because of the low initial resting potential, together with an increase in the electrotonic potential recorded in both cells. The initial coupling ratio was close to unity and at pH 6·4 it was no different. The time course of recovery of intracellular pH was similar to that seen at later stages of development.

The results of all experiments are collated in Table 2. They have been grouped according to the developmental stage, with the effect of treatment with different percentages of CO_2 also included. At the 4-cell and 8-cell stage treatment with 100% CO_2 never produced complete uncoupling although two of the 8 cell embryos showed some reduction in coupling ratio. At the 16-cell and 32-cell stage 100% and 80% CO_2 were as effective as at the morula and blastula stage, although 40% CO_2 had little effect on the coupling ratio. Once the embryo reached the 64-cell stage 40% and 100% CO_2 were equally effective. On occasion continuous recordings were made from the same embryo as it progressed from the 8-cell stage to the 64-cell stage. These embryos also demonstrated the stage dependent alteration in sensitivity of electrical coupling to treatment with carbon dioxide. The Table also illustrates two other features of the response. First, the variability of the depolarization: at early stages the membrane potential was relatively low, so that a smaller absolute change in membrane potential might be expected. But even at later stages, the depolarization

was extremely variable ranging from 0 to 31 mV. Secondly, the input resistance changes ranged from a small fall in input resistance to an over eightfold increase in input resistance. This variability is probably the consequence of two factors. Firstly, the sensitivity of the non-junctional membrane to CO_2 treatment may vary from embryo to embryo. Secondly the weakening of the intercellular seal normally provided by tight junctions during exposure to CO_2 (see p. 496) will introduce an additional shunt path to earth which will oppose any increase in input resistance at all stages of development. The first factor is probably responsible for the variable degree of depolarization produced by low intracellular pH.

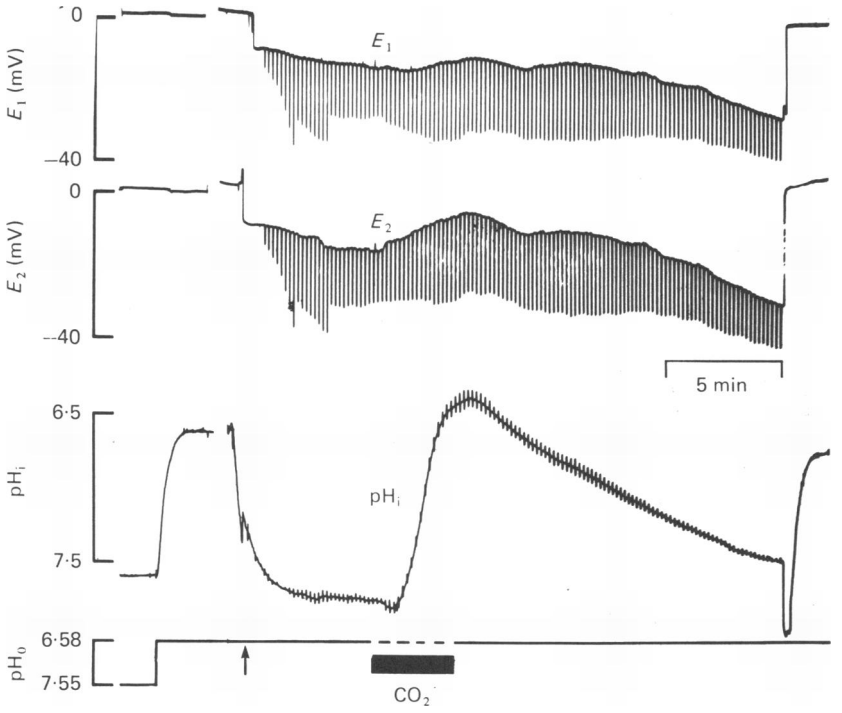

Fig. 5. 100% CO_2 does not abolish current flow from cell to cell in an embryo at the 4-cell stage. Arrow marks point of insertion of pH electrode. Note effect of CO_2 on intracellular pH very similar to that illustrated in Fig. 3. Current pulse 20 nA.

The relation between intracellular pH and electrical coupling between cells. Fig. 3 and 4 show that a small change in intracellular pH can have a profound effect on the degree of electrical coupling between cells of the early *Xenopus* embryo. This factor, together with the variability in intracellular pH from embryo to embryo made difficult the determination of the pH/coupling relationship from steady state determinations using fixed gas mixtures. Although the initial fall in pH produced by CO_2 treatment is likely to be limited by the response time of the pH-sensitive microelectrode, the slow time-course of the recovery of intracellular pH suggested that this phase might allow steady state measurements of pH_i and coupling. The point was checked by experiments of the type illustrated in Fig. 6.

The trace gives the continuous record of pH_i, the points measurements of coupling

TABLE 2

Developmental stage	E_1 mV initial	Coupling ratio initial	% CO_2	E_1 mV (CO_2)	Coupling ratio (CO_2)	Relative change in input resistance
4-cell (3)	16	0·9	100	1	0·9	1
4-cell (3)	6	0·95	100	0	0·95	1·2
*4-cell (3)	13	0·9	100	10	0·9	1·4
8-cell (4)	10	0·8	100	8	0·8	1
8-cell (4)	10	0·86	100	6	0·71	2·3
8-cell (4)	8	0·77	100	3	0·25	2
16-cell (5)	20	0·9	100	0	0·07	2·1
16-cell (5)	17	0·8	100	10	0	0·5
16-cell (5)	16	0·76	100	0	0	4
16-cell (5)	22	0·84	40	15	0·88	1·8
32-cell (6)	20	0·85	100	0	0	2·2
*32-cell (6)	42	0·64	100	17	0	4·3
32-cell (6)	45	0·54	80	11	0	>4
32-cell (6)	24	0·8	80	11	0	2·5
32-cell (6)	26	0·69	80	0	0	7·6
32-cell (6)	17	0·9	40	17	0·8	1·0
*32-cell (6)	30	0·85	20	16	0·65	8
32-cell (6)	11	0·9	20	7	0·9	1·6
64-cell (6½)	36	0·88	100	10	0	3
64-cell (6½)	16	0·9	100	0	0	1·7
64-cell (6½)	13	0·78	100	0	0	3
64-cell (6½)	30	0·7	100	0	0	1·8
64-cell (6½)	22	0·67	40	15	0·02	8·7
64-cell (6½)	15	0·44	40	8	0	17
*64-cell (6½)	24	0·65	40	12	0	3·3
128-cell (7)	20	0·87	100	5	0	2·3
128-cell (7)	31	0·6	100	0	0	>2·3

* Experiment illustrated in text.
Numbers in parenthesis give Nieuwkoop & Faber (1956) stage

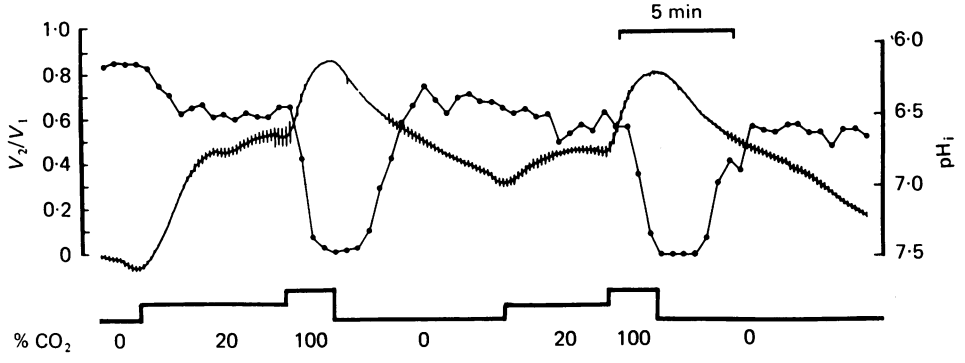

Fig. 6. Experiment to show that pH_i during return from exposure to 100% CO_2 is sufficiently slow for electrical coupling to be at a steady state. Embryo 32-cell stage. Continuous trace: record of pH_i taken during the experiment. Points: coupling ratio determined from pen records of the electrotonic potentials in adjacent cells obtained simultaneously. L.H. ordinate: Coupling ratio (V_2/V_1). R.H. ordinate: pH_i. The lower line indicates the periods of exposure of the embryos to 0, 20 and 100% CO_2.

ratio taken at intervals throughout the experiment from measurements of the electrotonic potentials recorded at E_1 and E_2. The initial pH was 7·62 and the coupling ratio 0·85. The bathing solution was then changed to a 20% CO_2/HCO_3^- buffered solution. This produced the usual fall in intracellular pH, reaching a steady value of 6·3 and a coupling ratio of 0·65. The external solution was then changed to 100% CO_2/HCO_3^- buffered solution and the intracellular pH dropped to 6·1; the coupling ratio fell to zero. After removal of CO_2 intercellular coupling gradually returned. When the pH_i reached 6·61, the value measured in 20% CO_2, the coupling ratio was 0·63, almost identical to that recorded in 20% CO_2. This confirms that the recovery of intracellular pH after removal of CO_2 is sufficiently slow for each interval to be considered equivalent to the steady state.

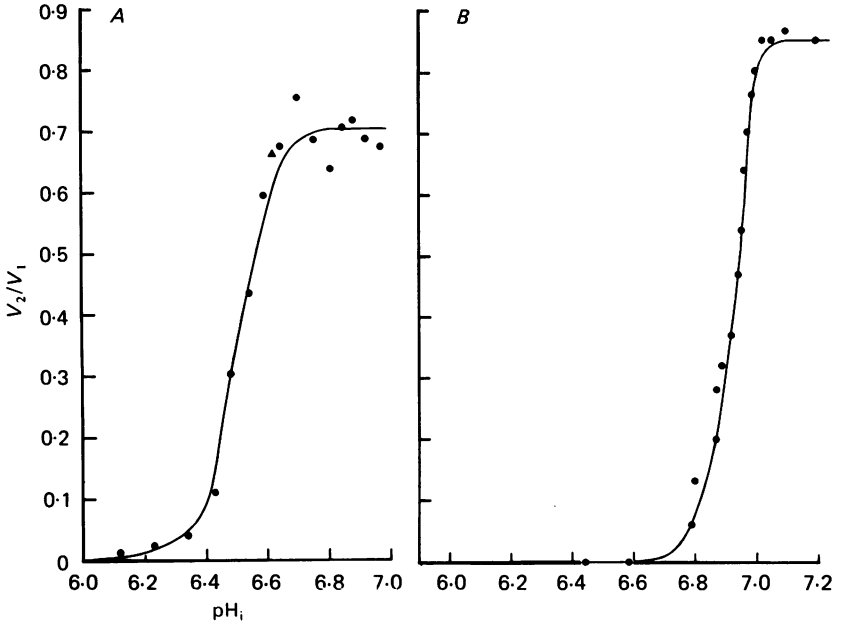

Fig. 7. The relation between intracellular pH and current flow from cell to cell. Ordinates: Coupling ratios (V_2/V_1). Abscissae: Intracellular pH. *A*, measurements made during the first recovery from 100% CO_2 treatment shown in Fig. 6; ▲, coupling ratio measured at steady state in 20% CO_2. ●, Coupling ratio measured during recoupling; *B*, Measurements made during first recoupling of Fig. 3.

Plots of the relationship between intracellular pH and coupling ratio were therefore constructed by measuring the coupling ratio at intervals during the recoupling process and plotting it against the appropriate value of pH_i. Fig. 7*A* shows such a plot derived from the early part of the experiment of Fig. 6. The steady state value measured in 20% CO_2 is indicated. Current began to flow into the adjacent cell once the intracellular pH reached 6·4. By pH 6·7 electrical coupling had returned and reached a steady ratio of 0·7. A similar plot, determined from the first recoupling illustrated in Fig. 3 is shown in Fig. 7*B*. Here electrical coupling between the cells did not appear until the intracellular pH reached 6·75. Complete return of current flow from one cell to the next occurred 0·2 pH units later, at pH 6·97.

Similar measurements in 6 other embryos all showed complete recoupling to occur over 0·2–0·4 pH units. The pH_i value at which recoupling commenced was not constant from embryo to embryo. Fig. 6*A* shows the most acid transition pH range seen; the most alkaline lay between 6·85 and 7·05.

It is not possible to derive the relationship between intracellular pH and junctional conductance from such plots. First, the coupling ratio is a function of both non-junctional and junctional membrane resistances. The depolarization produced by CO_2 treatment and the rise in input resistance at stages when uncoupling does not occur (see Table 2) both suggest that carbon dioxide has an effect on the non-junctional membrane resistance as well as the junctional resistance. Secondly, taking the simple 2-cell case, it can be shown that the positions of the pH/coupling ratio relationship and the pH/junctional conductance relationship are not identical. The position of the pH/electrical coupling relationship is governed by the magnitude of the coupling in the absence of CO_2. When the coupling ratio is good, it is little affected by sizeable alterations in junctional resistance. The lower the coupling ratio, the greater will be the effect of an alteration in junctional resistance. The pH/coupling ratio relationship always lies at more acid pH values than the pH/junctional conductance relationship, to a degree determined by the final coupling ratio. Thirdly, the measurements are complicated by the geometry of the preparation. At the 64-cell stage the embryo consists of two layers of electrically coupled cells, surrounding an eccentrically placed fluid-filled cavity. Current flows away from the injecting micro-electrode in two, if not three, dimensions. In this situation, the degree to which the measured electrotonic potentials reflect the behaviour of the junctional or non-junctional membrane pathway depends upon the inter-electrode spacing. When the inter-electrode spacing is small compared with the space constant of the preparation alterations in the magnitude of the electrotonic potential mainly reflect changes in the resistance of the intercellular pathway. If the inter-electrode spacing is close to the space constant then the behaviour of the electrotonic potential more closely reflects alterations in non-junctional membrane resistance. Before treatment with CO_2 the space constant is long compared with the inter-electrode spacing, but as uncoupling proceeds the space constant falls precipitously, so altering the sensitivity of the height of the electrotonic potential to variations in junctional resistance. The effect of this complication will be to reduce the sensitivity of the coupling ratio to alterations in junctional resistance when the coupling ratio is small (and therefore the space constant is short) so that the bottom end of the pH/coupling ratio relationship will be pushed to the right, thus compressing the pH range over which coupling ratio alterations take place.

The outcome of the considerations given above is that the relationship between intracellular pH and junctional conductance is probably less steep, and lies at more alkaline pH values than the measured pH/coupling ratio relationship. Quantitative statements about the relative positions of the two relationships cannot be made. The true junctional conductance/pH_i relation will have to await measurements on isolated pairs of cells where the electrical circuit can be completely analysed.

DISCUSSION

The results described in this paper show that the ease with which current spreads from one cell to the next in the early *Xenopus* embryo is greatly influenced by alterations in intracellular pH. Complete abolition of the direct cell to cell pathway occurs when the pH drops to between 6·9 and 6·5. The effect is rapidly reversible. The direct pathway does not become sensitive to alterations in intracellular pH until after the early cleavage stages.

Both the resting intracellular pH and intercellular pH recorded in early *Xenopus* embryos are high compared with values found in other systems. Early amphibian

embryos are generally poorly metabolically active and may generate very little metabolic acid at the stages studied here. Consequently the resting intracellular pH may initially be rather high; presumably as development proceeds the intracellular pH gradually falls. These findings may be of importance for *in vitro* studies of enzyme activities and for culture of cells isolated from early embryonic stages.

The finding that hydrogen ions are distributed between intra and intercellular phases according to the membrane potential is interesting because in most cells this is not the case, and hydrogen ions are supposed to be expelled from the cell interior via an active process (see Thomas, 1977). The situation with regard to the fluid bathing the embryo is less clear. The present experiments show that in the short term the pH of both intra-embryonic compartments is insensitive to alterations in the pH of the external fluid. Amphibian embryos normally develop in pond water, which is only weakly buffered. The impermeability of the external membrane of the outer cells may be sufficient to prevent leakage of hydrogen ions into the cells. Alternatively hydrogen ions may be pumped out of the cells at their external surfaces.

As in most preparations studied, treatment with carbon dioxide (e.g. Thomas, 1974) produces a prompt depolarization of the treated cells. The mechanism of this depolarization is not clear. The present measurements on cleavage stage embryos which do not uncouple when treated with CO_2, show a rise in input resistance, which suggests that the conductance of the non-junctional membrane falls during CO_2 treatment. Cells isolated from blastula stage embryos have a sizeable K^+ conductance (Slack & Warner, 1975). This conductance is reduced by treatment with CO_2 (L. Turin, unpublished results), which would explain the observed depolarization.

The question arises whether the effect of carbon dioxide on current flow between cells observed here is a general phenomenon. Following our preliminary report of these findings (Turin & Warner, 1977) similar results have been obtained in embryonic cells of *Fundulus* (Bennett, Brown, Harris, & Spray, 1978), *Ambystoma* (Hanna, Spray, Model, Harris & Bennett, 1978), *Chironomus* salivary gland (A. Warner & R. Wagner, unpublished; Rose & Rick, 1978) and electrically coupled cells of mammalian pancreatic and lacrimal acini (Iwatsuki & Petersen, 1979). It seems likely therefore that the intercellular pathway in most communicating systems will be sensitive to carbon dioxide. The effective agent is probably the intracellular pH, rather than anoxia, since our results show that 40% CO_2/60% O_2 mixtures are as effective as 100% CO_2; Iwatsuki & Petersen (1979) have reached a similar conclusion.

The results raise two other major questions. First, why does acidification of the intracellular pH not reliably abolish electrical coupling until the 64-cell stage of development? One possibility is that small intercellular cytoplasmic bridges formed by incomplete cleavages remain between some cells, even on occasion as late as the 32-cell stage. Alternatively the properties of the intercellular junction may alter when the embryo enters the morula stage.

Second, how does the H^+ ion exert its effect on junctional permeability? Considerable evidence has accumulated to suggest that the intracellular level of ionized Ca (a^i_{Ca}) can powerfully influence the permeability of the intercellular junction, an increase in (a^i_{Ca}) reducing the flow of ions and other molecules from one cell to the next (Loewenstein, Nakas & Socolar, 1967; Rose & Loewenstein, 1976). This ability is shared by Sr^{2+} (Oliveira-Castro & Loewenstein, 1971; Mello, 1975) and Mg^{2+},

Ba^{2+} and Mn^{2+} (Oliveira-Castro & Loewenstein, 1971). In the *Chironomus* salivary gland the selectivity sequence was $Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$, with Ca about 10 times more effective than Mg; the minimum effective dose of Mn^{2+} was not determined. In heart muscle (Mello, 1975) Ca^{2+} and Sr^{2+} were about equally effective, but Mg^{2+} (at the same concentration) was ineffective. These findings suggest that the site controlling junctional permeability does not form a highly specific coordination complex with Ca^{2+}, and one would therefore expect, on physico-chemical grounds alone (see for example Schubert, 1955), H^+ ions to interact with these charged groups. The observed pH dependence of the uncoupling gives no clue to the identity of the relevant membrane group because the pK_a is likely to be shifted from its position in free solution by interactions with neighbouring membrane sites. The situation is further complicated by interactions between H^+ and Ca^{2+} within the cell. When the intracellular pH falls it is likely that Ca^{2+} ions will be displaced from intracellular binding sites and mitochondria; a rise in intracellular Ca^{2+} produces a concomitant fall in intracellular pH (Meech & Thomas, 1977). These considerations raise three possibilities: (i) secondary effects of Ca^{2+} and H^+ on electrical coupling are superimposed upon the direct effects of alkaline earths and hydrogen on the junctional membrane sites; (ii) uncoupling is always achieved by Ca^{2+} ions with H^+ ions merely releasing Ca^{2+} from intracellular sequestering sites; (iii) uncoupling is always achieved by H^+, with Ca^{2+} and the alkaline earth metals releasing H^+ from intracellular buffers. How far does the present evidence allow us to distinguish between these possibilities?

Baker & Honerjäger (1978) showed in the squid axon that a fall in intracellular pH to 6·7 reduced intracellular ionized Ca, while Lea & Ashley (1978) found ionized Ca levels in barnacle muscle to rise during treatment with 100% CO_2. On the one hand, Rose & Rick (1978), working on *Chironomus* salivary gland, found CO_2 induced uncoupling to be associated with a rise in free intracellular Ca on ten out of twenty-five occasions. On the other hand, Bennett *et al.* (1978) could not record any increase in a^i_{Ca} associated with uncoupling brought about by a low intracellular pH in embryonic cells of *Fundulus*. Iwatsuki & Petersen (1978) concluded that acetylcholine induced uncoupling of cells in pancreatic acini was probably the consequence of an increase in intracellular Ca^{2+}, yet their study with CO_2 showed the efficacy of ACh induced uncoupling to be altered by changing the intracellular pH (Iwatsuki & Petersen, 1979). Evidence to suggest that Ca^{2+} and H^+ can independently influence the properties of the gap junction comes from the work of Peracchia (1978) and Peracchia & Peracchia (1978). At pH 7·5, 5×10^{-7} M-Ca^{2+} produces alterations in the ultrastructure of isolated gap junctions similar to those seen between cells which have been uncoupled from each other by Ca^{2+} (Peracchia, 1978). Lowering environmental pH to 6·5 or below, in the absence of Ca^{2+}, produces alterations in the packing of gap junction particles consistent with the view that H^+ can independently uncouple cells (Peracchia & Peracchia, 1978).

This confusing literature suggests that Ca^{2+} and H^+ each have both direct and secondary effects on junctional permeability. The degree to which intracellular Ca^{2+} and H^+ influence the concentration of each other through release from sequestering sites depends on the relative degree and speed of Ca^{2+} and H^+ buffering in a particular preparation. It will not be easy to separate these interrelated effects.

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